

## Identification and characterization of a circadian clock-associated pseudo-response regulator 7 gene from trifoliolate orange

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### Abstract

Circadian clock is usually involved in many physiological processes of plants, including responses to abiotic stress, whilst pseudo-response regulator 7 (PRR7) gene is the main component of the circadian clock. In this study, the cDNA of the PRR7 gene was obtained from trifoliolate orange (*Poncirus trifoliata*). Based on the sequence analysis, the PtPRR7 gene had an open reading frame of 2343 bp, encoded 780 amino acids, and contained proteins of the REC and CCT domains. Subcellular localization indicated that PtPRR7 was mainly localized in the nucleus and a small amount of cytoplasm. qRT-PCR analysis revealed the highest expression level of *PtPRR7* in roots than in both shoots and leaves. The *PtPRR7* gene during 24 hours of soil water deficit exhibited a circadian rhythmic expression pattern: the expression peak at 9:00 am in leaves and at 21:00 pm in roots. Drought treatment affected *PtPRR7* gene expression. Such data provide important references for understanding the characteristics of *PtPRR7* gene in citrus plants.

**Keywords:** citrus; diurnal rhythm; drought; pseudo-response regulator

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### Introduction

Due to the rotation of the earth, there are daily changes in the surrounding environment of plants, mainly including light and temperature (Bhattacharya *et al.*, 2017). To adapt to this environment, plants must respond with a circadian clock (Nohales and Kay, 2016). Meanwhile, the circadian clock regulates the synthesis and utilization of resources in a transient manner, and ultimately enhances the plant's adaptability and resistance to stress (Seo and Mas, 2015). Circadian clock usually controls many important physiological processes of plants, such as hypocotyl elongation, leaf movement, stomatal switching, flowering, etc. (Greenham and McClung, 2015). It can enable organisms to anticipate fluctuations in the external environment, coordinate vital physiological activities and developmental processes at appropriate times of the day, and improve adaptability to the environment (Yerushalmi and Green, 2009).

In plants, the transcription can be regulated by the circadian clock (Covington *et al.*, 2008; Michael *et al.*, 2008). Liu *et al.* (2013) observed that the principal component of the circadian clock in *Arabidopsis* was pseudo-response regulator 7 (PRR7) directly involved in the inhibition of plant growth, light signals and stress responses. Moreover, the *PRR7* expressions are conserved among different plant species (Khan *et al.*, 2010; Filichkin *et al.*, 2011). Components of circadian clock interacted to establish a complex network of feedback loops at the transcription level (De Caluwé *et al.*, 2016). Two homologous MYB transcription factors, circadian clock associated 1 (*CCA1*) and late elongated hypocotyl (*LHY*), peaked near dawn to activate *PRR9* and *PRR7* expressions (Farré *et al.*, 2005). *PRR5*, *PRR7* and *PRR9* protein levels peak and end a day, thereby inhibiting *CCA1* and *LHY* expressions (Farré and Kay, 2007; Nakamichi *et al.*, 2010). The mutant having *PRR5*, *PRR7*, and *PRR9* was more resistant to drought and cold, with the increased abscisic acid (ABA) levels (Fukushima *et al.*, 2009; Syed *et al.*, 2015). As a result, *PRR*-related genes are strongly correlated with drought tolerance (Juliana *et al.*, 2014; Li *et al.*, 2017).

Citrus is one of the most widely cultivated fruit crops (Wu *et al.*, 2013, 2019), which often suffers from soil water deficit during summer and autumn, thereby, causing poor vigor growth and low fruit quality (Poggi *et al.*, 2007; Osakabe *et al.*, 2013; Santana-Vieira *et al.* 2016; He *et al.*, 2020). Trifoliate orange (*Poncirus trifoliata* L. Raf.) is an important rootstock of citrus plants used in Southeast Asia and also extreme drought-sensitive plant (Zhang *et al.*, 2020). Studies on circadian clock genes are focused on model plants such as *Arabidopsis thaliana* and rice (Garg *et al.*, 2019; Jain *et al.*, 2018), while the information regarding the *PRR7* gene in trifoliate orange is scarce. The objective of the present work is to clone the *PRR7* gene from trifoliate orange and also analyze the characteristics and the functions of the gene in response to drought stress.

## Materials and Methods

### *Cloning and sequence analysis*

Leaves of trifoliate orange were ground with liquid nitrogen. Total RNA was extracted using the kit PrimeScript™ RT Master Mix (Baori Medical Biotechnology Beijing Co., Ltd., China), and reversely transcribed into cDNA using the kit PrimeScript™ RT reagent Kit with gDNA Eraser (Baori Medical Biotechnology Beijing Co., Ltd., China). Specific primers for PCR amplification were developed according to the sweet orange database (<http://citrus.bzau.edu.cn/orange/index.php>) (Table 1). The RT-PCR products were purified and connection to the 1300-35S-YFP vector. The ligation product was transformed into *Escherichia coli*, and the positive clones were chosen for sequencing. Sequence alignment was carried out using the DNAMAN (version 5.2.2) program. A phylogenetic tree was constructed using MEGA-X for the amino acid sequences of the circadian clock gene from different plants. The phylogenetic tree generation algorithm was obtained by the Neighbor-joining (NJ) method (Saitou and Nei, 1987). We used the bootstrap to verify the phylogenetic tree and define the number of repetitions to 1000 (Sitnikova *et al.*, 1995). Structural prediction of the gene was calculated as per the SWISS-MODEL online tool (<https://swissmodel.expasy.org/>).

### *Subcellular localizations of PtPRR7*

The subcellular localization vector was designed to construct primers PtPRR7-F/R (Table 1) and a target band was used as a template to amplify the target band. The recombinant method was inserted into the EcoRI / SpeI site of the 1300-35S-YFP vector to construct a satisfactory subcellular localization vector (PtPRR7RRFP) to transform *Agrobacterium* EHA105. The transient expression method was utilized to inject *Agrobacterium* that successfully transformed the target plasmid into tobacco leaves, and cultured for 48 hours in the dark. Using the 3302Y3 empty vector as a control, the laser confocal microscope (Leica SP-5, Leica, Wetzlar, Germany) was used to observe the distribution of YFP fluorescence in the cells.

**Table 1.** Genes-specific primer sequences used in this study

Primer names	Primer sequence (5'→3')	Purpose
<i>PtPRR7</i>	F: aagagacaggatccgaattcatggACGTTGATGGCAAAGCAGAC	Vector construction of Subcellular localization
	R: acctccgaccggtgcactagtAGAACATTCTGGTTCCCTGC	
<i>PtPRR7</i>	F: TAGGAGCACACAAGAGCAGC	qRT-PCR and qPCR
	R: TTGTGGAACAGCTTCAGCCA	
<i>β-Actin</i>	F: CCGACCGTATGAGCAAGGAAA	Reference gene for qRT-PCR
	R: TTCCTGTGGACAATGGATGGA	

*Plant cultures and drought treatments*

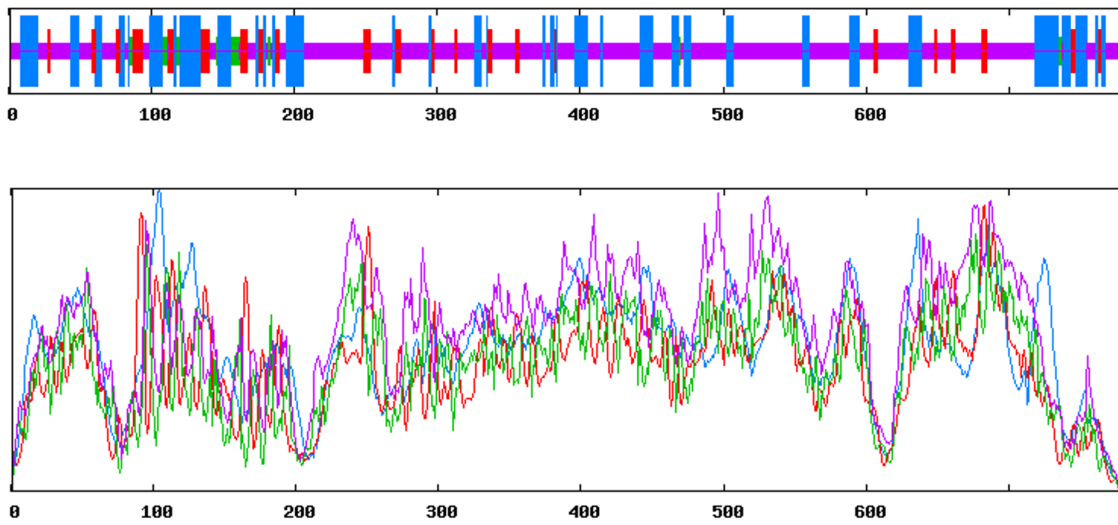
Seeds of trifoliolate orange were surface-sterilized with 70% alcohol solutions for 10 minutes, washed five times with distilled water, and germinated in sterilized (121 °C, 0.1 MPa, 1 h) river sand in a light incubator. The environmental conditions were set to a temperature of 28 °C / 20 °C (16 h day / 8 h night), a light intensity of 1200 Lux, and a constant relative humidity of 68%. Three trifoliolate orange seedlings having five leaves were transplanted into a plastic pot supplied with autoclaved substrate growth substrate (soil:sand = 5:1 v/v). These plants were grown in a greenhouse without any temperature control equipment. The soil water of the pots was regularly maintained in 75% of the maximum soil water holding capacity for 11 weeks. Subsequently, 18 pots were kept under 75% of the maximum soil water holding capacity conditions, while the other 18 pots were subjected to 55% of the maximum soil water holding capacity for 11 weeks. Soil moisture was controlled by daily weighing with an electronic scale. In order to analyze the response pattern of *PtPRR7* to drought stress, nine seedlings from three pots were kept in the target soil water content and then harvested every four hours from 9:00 am of the first day to 5:00 am of the next day, and a total of six harvests were done.

*qRT-PCR analyses*

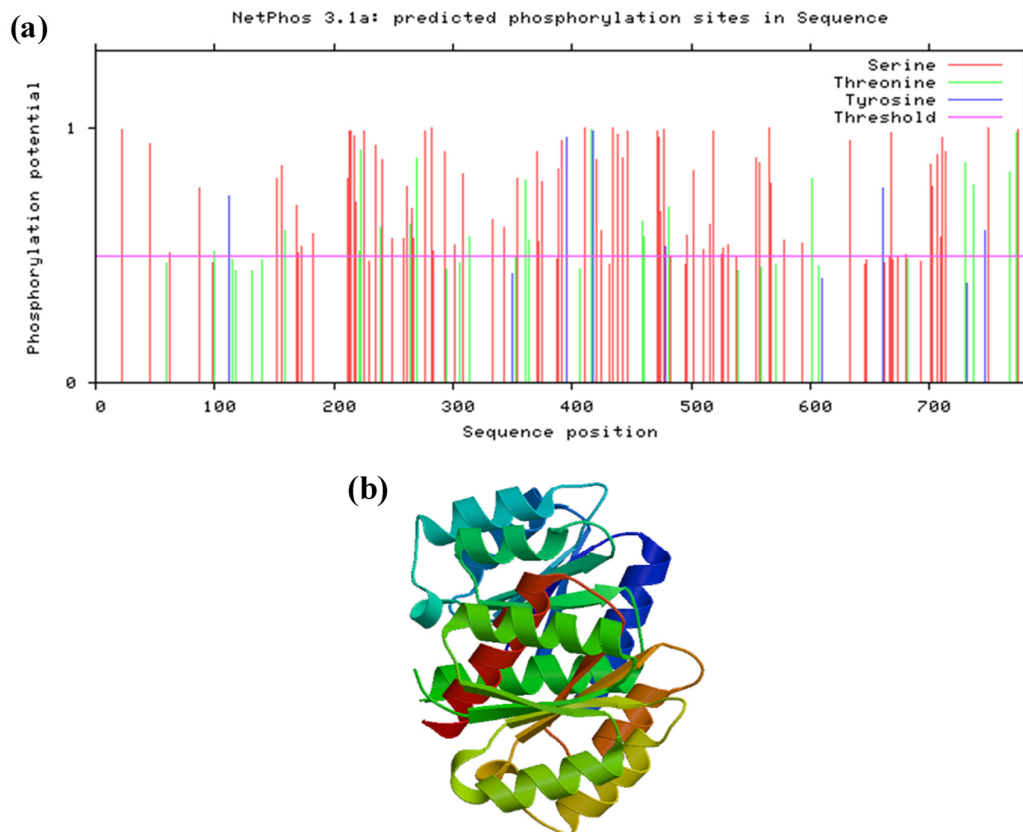
Total RNA of leaves and roots was extracted and the cDNA was synthesized as indicated in 2.1 Cloning and sequence analysis. qRT-PCR analysis was performed using the ChamQ™ Universal SYBR qPCR Master Mix Kit (Vazyme Biotech Co., Ltd). The qRT-PCR primers were developed based on the sequences obtained (Table 1). The qRT-PCR reaction was previously described by He *et al.* (2019). CFX96 real-time PCR detection system (BioRad Laboratories, USA) was used. Each sample had three biological replicates with three technical replications. The relative quantification was calculated using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001), and  $\beta$ -actin was used as a reference gene to normalize gene expression.

**Results***Acquisition of PtPRR7 full-length and bioinformatical analysis*

The *PtPRR7* gene (GenBank accession No: MN890019) had an open reading frame of 2343 bp and encodes 780 amino acids. Its molecular weight is 84845.39 kDa and its isoelectric point is 7.54. The protein instability index was 49.28, and the average hydrophobicity was -0.731. The first three amino acid contents were alanine (31.7%), glycine (25.7%) and threonine (23.4%), and the fat index was 71.50. At the same time, secondary structure predictions revealed that approximately 62.31% of the *PtPRR7* protein sequence had random coils and 9.87% extended strands, and the  $\alpha$ -helix and  $\beta$ -turn were 25.38% and 2.44%, respectively (Figure 1). The NetPhos (<http://www.cbs.dtu.dk/services/>) predicted 52 serine, 10 threonine and 5 tyrosine in the *PtPRR7* protein as potential phosphorylation sites (Figure 2a). Molecular simulation indicated that the predicted three-dimensional structure of the *PtPRR7* protein consisted of a two-component system response regulator and a GDGEF domain protein (Figure 2b).

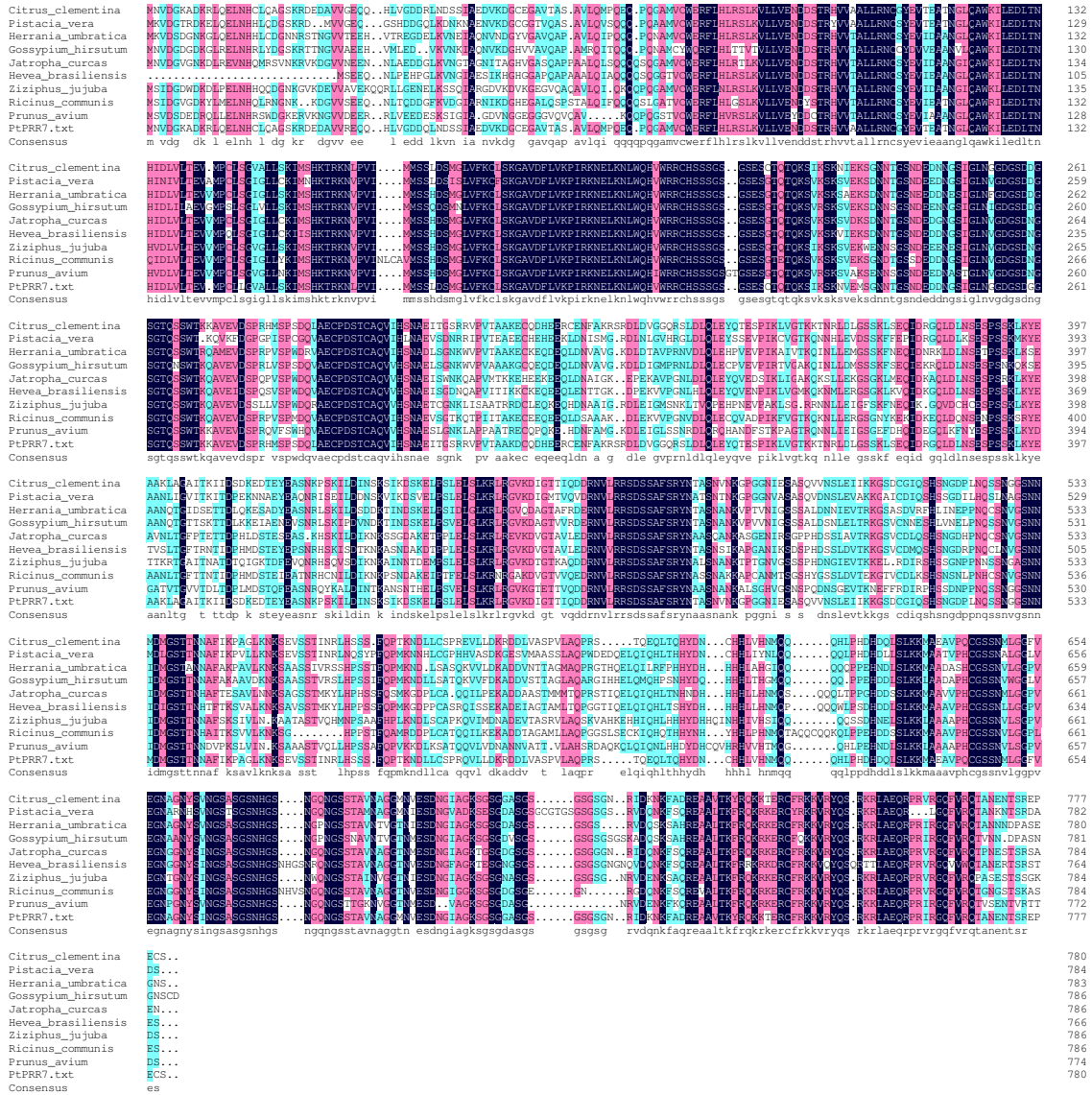


**Figure 1.** Distribution of the secondary structure of the *PtPRR7* gene. The vertical lines in the picture above are  $\alpha$ -helix (blue), extended strand (red),  $\beta$ -sheet (green), and random curl (purple)



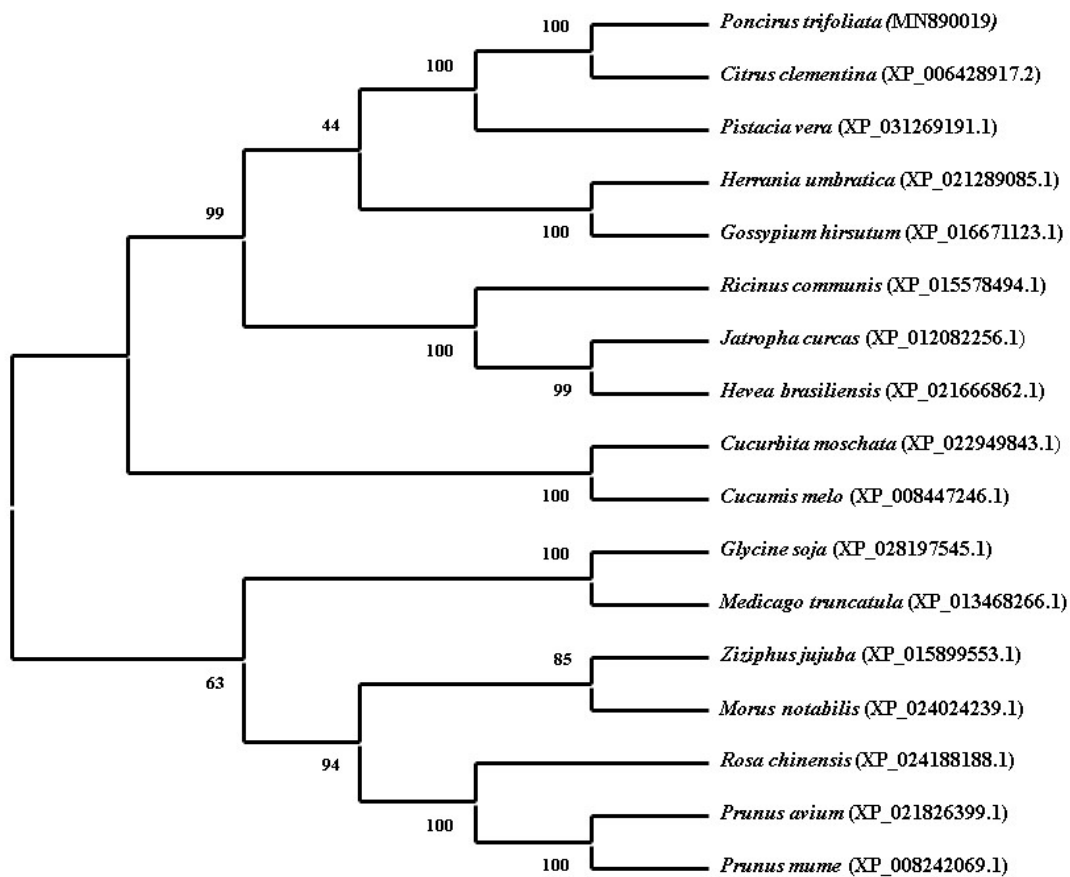
**Figure 2.** Analysis of amino acid sequences (a) and three-dimensional structure (b) of *PtPRR7* protein

The amino acid sequence of the *PtPRR7* protein was compared with the amino acid sequence of PRR7 protein of nine additional plant species by DNAMAN. The results showed that the sequence homology of PRR7-type proteins from different plant families was 75.54% (Figure 3), indicating that the PRR7-type protein has high sequence conservation.



**Figure 3.** Multiple alignments of the amino acid sequence of *PtPRR7* with other PRR7 family proteins. Conserved amino acid residues in all proteins are shaded in black. Red shading indicates a similarity of 75% or more, and blue shading indicates an acquaintance of 50% or more

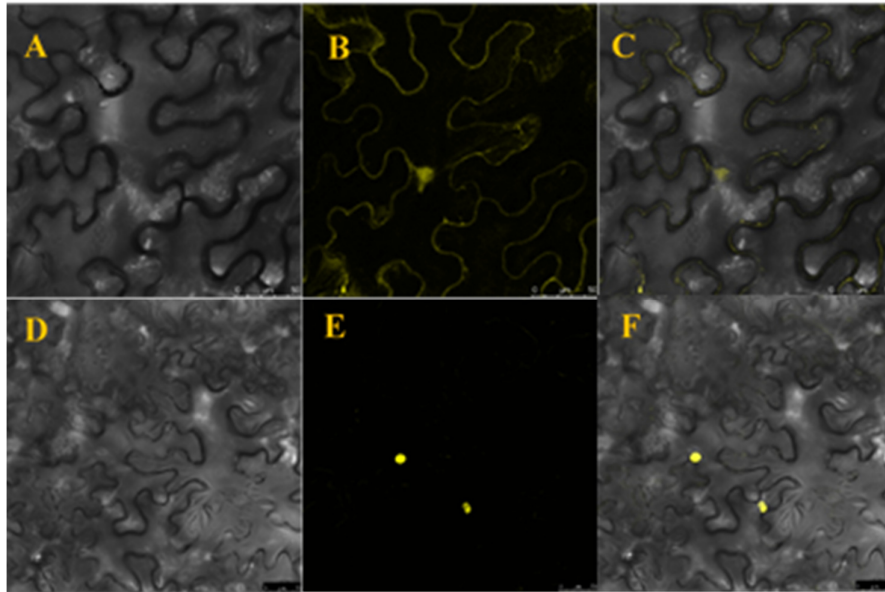
The phylogenetic relationship between PRR7 and its homologues indicated that all proteins had different clusters. *PtPRR7* is grouped with the member of *Citrus clementina*, and is closest to the PRR7 of *Pistacia vera* (Figure 4). And the branch of the phylogenetic tree showed that the PRR proteins of the dicotyledonous and monocotyledonous plants were branched into two groups.



**Figure 4.** Phylogenetic relationship between *PtPRR7* constructed with MEGA-X software and the homologs of other plants

#### *Subcellular localizations of PtPRR7 gene*

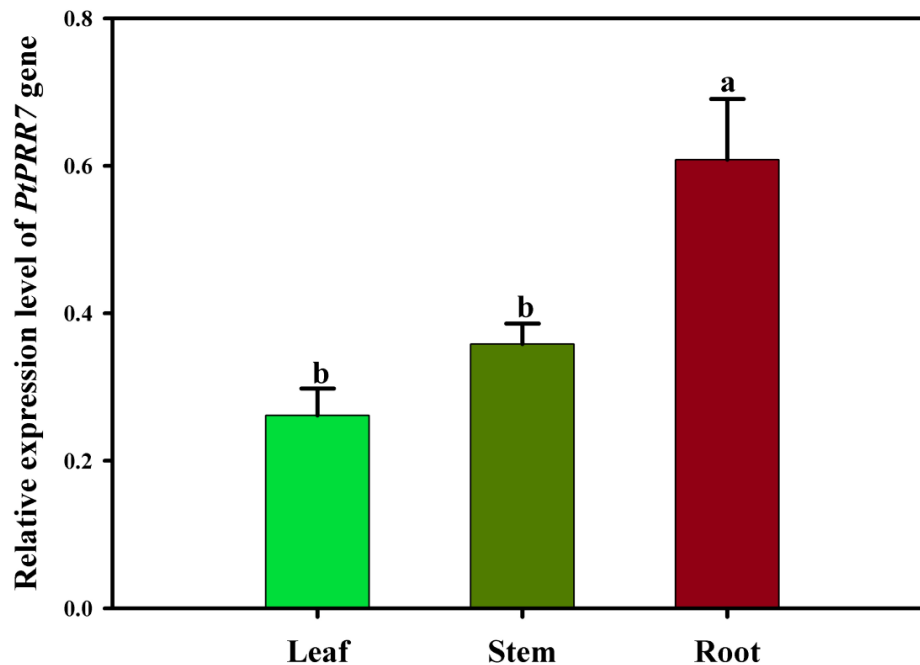
The results of subcellular localization showed that *PtPRR7* was localized in the nucleus. In order to analyze the subcellular localization of the *PtPRR7* protein, a 35 S:*PtPRR7*:YFP vector was constructed, with 35S :: YFP empty as a control. After the plasmid was successfully transformed into *Agrobacterium*, the leaves of Bunsen tobacco were injected. After 48 hours of dark culture, the yellow fluorescent protein distribution was observed under Leica SP-5 laser confocal microscopy. As shown in Figure 5, it was found that the *PtPRR7* gene is mainly located in the nucleus, and there is a weak signal on the plasma membrane. Combined with the prediction results, *PtPRR7* has a nuclear localization signal and homologous genes can function as transcription factors. It is speculated that *PtPRR7* is also located in the nucleus as a transcription factor and plays a role in transcription regulation.



**Figure 5.** Subcellular localization of *PtPRR7*. A–C: the positive control of 35S::YFP. D–F: 35S:: *PtPRR7*: YFP fusion protein result. A and D: bright field; B and E: YFP; C and F: merged

*Tissue-specific expression patterns of PtPRR7 gene*

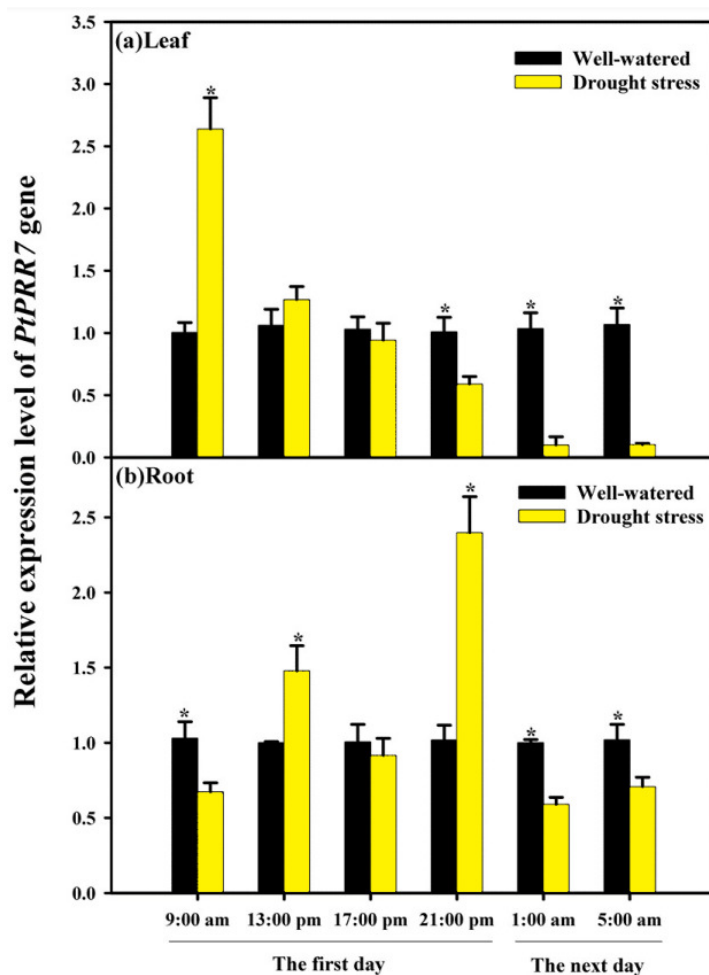
The tissue expression profile of *PtPRR7* was detected using qRT-PCR. The results showed that *PtPRR7* could be expressed in roots, stems and leaves (Figure 6). The expression level of *PtPRR7* in roots was significantly 1.7-times and 2.3-time higher than that in stems and leaves, respectively.



**Figure 6.** The tissue-specific expression of *PtPRR7* gene. Data (means  $\pm$  SD,  $n = 3$ ) are significantly different ( $P < 0.05$ ) if followed by different letters above the bars

*Expression patterns of PtPRR7 in response to drought stress*

Time-specific expression pattern of *PtPRR7* gene showed strong periodicity, with obvious peaks and troughs (Figure 7). During 24 hours of soil water deficit between 9:00 am of the first day to 5:00 am of the next day, the expression of *PtPRR7* gene in leaves reached its peak at 9:00 am of the first day. The expression level of *PtPRR7* gene in root reached its peak at 21:00 pm of the first day. Compared with well-watered treatment, drought stress treatment induced an up-regulated expression of *PtPRR7* in leaves at 9:00 am of the first day and a down-regulated expression of *PtPRR7* in leaves at 21:00 pm of the first day, 1:00 am of the next day, and 5:00 pm of the next day (Figure 7a). Additionally, in roots, drought treatment induced the expression of *PtPRR7* at 13:00 am and 21: am of the first day while down-regulated the expression of *PtPRR7* at 1:00 am and 5:00 am of the next day (Figure 7b). At the same time, the expression of drought treatment at other time points was lower than that of normal water. During 24 hours of drought stress, the expression level of *PtPRR7* gene in leaves and roots was the lowest at 1:00 am of the next day.



**Figure 7.** Time-specific expression patterns of *PtPRR7* gene in leaf (a) and root (b) in response to well-watered and drought stress conditions. Data (means  $\pm$  SD,  $n = 3$ ) are significantly different ( $P < 0.05$ ) if followed by asterisks (\*) above the bars

## Discussion

In this study, the PRR7 gene was firstly cloned in trifoliolate orange. The comparison of sequencing showed that the PRR7 gene had conserved REC and CCT domains at the N- or C-terminus of the amino acid sequences of the PRR genes in other plants. This is in agreement with other plants (Makino *et al.*, 2000; Farré and Liu, 2013). The phylogenetic tree showed that the PRR protein of dicotyledonous and monocotyledonous plants formed two groups that are far. PtPRR7 is closest to PRR7 of *Citrus clementina*. Obviously, the PRR gene of citrus is evolutionarily conserved as a congeneric gene (Murakami *et al.*, 2007; Takata *et al.*, 2010).

Previous studies indicated that acidic isomers of PRR proteins in tobacco, such as PRR-1a, PRR-1b, and PRR-1c, existed in extracellular space (Dixon *et al.*, 1991; Elvira *et al.*, 2008; Riviere *et al.*, 2008). Our results showed that PtPRR7 is a slightly acidic protein with an isoelectric point of 7.54. On the other hand, it is consistent that PtPRR7-YFP has a small amount of fusion outside the cell. Some literatures indicate that PRR is involved in the regulation of transcription factors and transcription inhibitors (Para *et al.*, 2007), and most transcription factors are also localized in the nucleus. Subcellular localization results showed that the protein expressed by *PtPRR7* was mainly localized in the nucleus, which was consistent with the expected results. qRT-PCR analysis revealed that the *PtPRR7* gene was expressed in roots, stems, and leaves, whilst the *PtPRR7* gene was the highest expression in roots. The results are in agreement with a study in mulberry (Fang *et al.*, 2019).

Time-expression analysis showed that the *PtPRR7* gene had the circadian feature in leaves and roots of trifoliolate orange during drought stress, which agrees with earlier studies in Arabidopsis and turnip (Kim *et al.*, 2007). Our results also indicated that the expression of *PtPRR7* gene in leaves during drought stress peaks at 9:00 am, because PRRs family genes are expressed in the morning (Wang *et al.*, 2013). Interestingly, the expression of *PtPRR7* gene in roots peaked at 21:00 pm. In Arabidopsis, the sensitivity of PRRs was linked to light (Farré and Liu, 2013). PRR7 gene regulated the upstream regions of several genes involved in ABA signaling pathways, a mechanism of drought tolerance (Chen *et al.*, 2008; Du *et al.*, 2012; Wang *et al.*, 2018). Therefore, the *PtPRR7* gene may improve drought tolerance by regulating genes implicated in ABA signaling pathways, which have yet to be studied. In addition, it has been noted that the expression of PRR genes was induced by other abiotic stress, besides drought (Atamian and Harmer, 2016; Nakamichi *et al.*, 2016).

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## Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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